



## Review

## Influence of the scaffolding protein Zonula Occludens (ZOs) on membrane channels ☆


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## ABSTRACT

Zonula Occludens (ZO) proteins are ubiquitous scaffolding proteins providing the structural basis for the assembly of multiprotein complexes at the cytoplasmic surface of the plasma membrane and linking transmembrane proteins to the filamentous cytoskeleton. They belong to the large family of membrane-associated guanylate kinase (MAGUK)-like proteins comprising a number of subfamilies based on domain content and sequence similarity. ZO proteins were originally described to localize specifically to tight junctions, or *Zonulae Occludentes*, but this notion was rapidly reconsidered since ZO proteins were found to associate with adherens junctions as well as with gap junctions, particularly with connexin-made intercellular channels, and also with a few other membrane channels. Accumulating evidence reveals that in addition to having passive scaffolding functions in organizing gap junction complexes, including connexins and cytoskeletons, ZO proteins (particularly ZO-1) also actively take part in the dynamic function as well as in the remodeling of junctional complexes in a number of cellular systems. This article is part of a Special Issue entitled: Reciprocal influences between cell cytoskeleton and membrane channels, receptors and transporters. Guest Editor: Jean Claude Hervé.

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Abbreviations: 42GPA9, mouse Sertoli cell line;  $\beta$ TC-3, mouse pancreatic  $\beta$  cell line derived from insulinomas; COS7, monkey African green kidney cells; HEK293, human embryonic kidney 293 cells; MDCK, epithelial Madin–Darby canine kidney cells; N/N1003A, rabbit lens epithelial cells; NRK, rat kidney cells; PC-12 cell, a cell line substitute neuron, originally cloned from rat pheochromocytoma cells; ROS 17/2.8, rat osteosarcoma cells

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## 1. Introduction

Zonula occludens (ZO) proteins are ubiquitous scaffolding proteins providing the structural basis for the assembly of multiprotein complexes at the plasma membrane. They belong to the large family of membrane-associated guanylate kinase (MAGUK)-like proteins comprising a number of subfamilies based on domain content and sequence similarity. Fanning and Anderson [1] hypothesized that MAGUK proteins would cluster ion channels, receptors, adhesion molecules and cytosolic signaling proteins at specific membrane domains and tightly link these proteins to the cytoskeleton. Such cytoskeletal docking restricts ion transport proteins (channels, transporters) to specialized domains of the plasma membrane. ZO proteins, present only in multicellular organisms, associate among themselves and with other integral and adaptor proteins of the tight junctions, of the adherens junctions and of gap junctions, as with numerous signaling proteins and the actin cytoskeleton.

Protein–protein interactions occurring via the recognition of short peptide sequences by modular interaction domains play a central role in the assembly of signaling protein complexes and larger protein networks that regulate cellular behavior. ZO proteins, comprising ZO-1, -2, and -3, are ubiquitous peripheral proteins localizing at junctional sites. ZO proteins are multi-domain scaffolds that bind directly various types of proteins to the cytoplasmic surface of the junction and connect (either directly or indirectly) transmembranous junctional proteins with the actin cytoskeleton. ZO proteins were originally described to localize specifically to tight junctions (TJs or *zonulae occludentes* [2]) but this notion was rapidly reconsidered since these proteins were found to associate with the cadherin-based adherens junctions in cells lacking TJs, then later with gap junctions (GJs), particularly with connexin-made intercellular channels (*for review, see* [3]) and with a few membrane channels. In GJs, accumulating evidences indicate that in addition to having passive scaffolding functions in organizing GJ complexes, including connexins and cytoskeletons, ZO proteins, particularly ZO-1, also actively take part in the dynamic function as well as in remodeling of GJs in a number of cellular systems.

## 2. The Zonula Occludens protein family

ZO proteins belong to the superfamily of MAGUK-like proteins, which comprises a number of subfamilies based on domain content and sequence similarity and plays essential roles in organ development, cell–cell communication, cell polarity establishment and maintenance, and cellular signal transduction. ZO-1, with a molecular mass of 220 kDa, was the first TJ-associated protein to be identified. ZO-1 associates with ZO-2, a 160 kDa protein and the 130 kDa protein ZO-3. The three ZO proteins have been inactivated in mice; ZO-3 knockout mice lack an obvious phenotype whereas animals deficient in ZO-1 or ZO-2 show early embryonic lethality.

## 3. Protein–protein interaction modules of ZOs

ZO proteins have an N-terminal half with a domain structure similar to other MAGUK proteins, characterized for presenting a core of three domains: (i) PDZ, an acronym combining the first letters of three proteins which were first discovered to share the domain: the post synaptic density protein 95 (PSD95) also known as synapse-associated protein 90 (SAP90), the *Drosophila* septate junction protein Discs-large (Dlg), and the epithelial tight junction protein Zonula occludens-1, (ii) SH3 (the Src homology 3 domain) and (iii) guanylate kinase (GuK).

### 3.1. PDZ domains

Although originally identified in metazoans, PDZ domains have been found to be spread through bacteria, fungi and plant lineages

as well (*for review, see* [3]). PDZ domains (80–100 amino acid residues) play a key role in cellular signaling. The second PDZ domain of ZO proteins is responsible for the homo and hetero dimerization of these molecules [4,5]. The most common function of PDZ domains is their ability to bind to a short stretch of amino acid residues at the C-termini of target proteins. While their primary sequence can differ markedly, the three-dimensional structure of PDZ domains is remarkably conserved. The PDZ domain groove ends with a hydrophobic cavity to which the predominantly, but by no means exclusively, hydrophobic side chain of the C-terminal end residue of the partnering protein inserts. Indeed, the nature of this pocket appears to have considerable influence on the specificity of partnerships that a particular PDZ domain can undergo (*see* [6]). Moreover, certain PDZ domains appear to be able to bind phosphatidylinositol 4,5-bisphosphate (commonly known as PIP<sub>2</sub>), and cyclic peptides [7], leading to the possibility for some small molecules and biologics to be able to manipulate PDZ domain functioning.

### 3.2. SH3 domain

The Src homology 3 domain is a small (about 60 amino acid residues), non-catalytic protein module shared among diverse signaling and cytoskeletal proteins of eukaryotes. The best-characterized function of SH3 domains is to bind proline-enriched sequences of the type P-x-x-P (x, any residue), mediating the assembly of large multi-protein complexes.

### 3.3. GuK domain

GuK (guanylate kinase) domains exhibit sequence similarity to guanylate kinase, which converts GMP to GDP using ATPase as a phosphate donor, but several studies suggest that the GuK domain of MAGUK proteins has evolved into a protein–protein binding domain. Ca-calmodulin for example was found to bind to the GuK domain of ZO-1 [8], leading Fanning and Anderson [9] to suggest that intracellular calcium levels might control some ZO-1 functions.

The MAGUK SH3 domain frequently shows an atypical binding specificity, it does not bind to proline-rich sequences but instead forms an internal structure supramodule via an intramolecular interaction with the subsequent GuK domain [10,11]. This SH3-hinge-GuK unit is unique among MAGUK proteins. The sequence is indeed much longer than 30 other MAGUKs and not homologous to these (*see* [12]). According to these authors, proteins with different regulatory potential (e.g. G-proteins and G-protein regulators) might adhere to and influence cellular functions of ZO-proteins, and the interactions can be modulated via its hinge region and/or the binding proteins.

ZOs also carry a proline-rich region located either at the C-termini (ZO-1, ZO-2) or between the second and third PDZ domain (ZO-3) [13]. Positioned between the conserved domains of MAGUKs are highly variable sequences termed “unique” (U) motifs; starting from the N-terminus these are Unique-1 (U1), followed by U2 between the first and second PDZ domains, etc.

The C-terminal regions are unique to the three ZOs and not present in MAGUK proteins such as PSD95, calcium/calmodulin-dependent serine protein kinase (CASK), and Dlg. Although highly divergent at the amino acid level, the C-terminal regions all bind directly to F-actin or with other actin-binding proteins, such as protein 4.1 and cortactin [14].

## 4. Interactions of Zonula Occludens proteins with membrane channels

The members of the superfamily of transient receptor potential (TRP) cation channels are involved in a plethora of cellular functions. One member of the “canonical” subfamily *TRP channels*, TRPC4, was found to interact with the PDZ1 domain of ZO-1 [15]. A “nearly perfect”

**Table 1**

Reported interactions of channel proteins with ZO proteins.

Partner protein	Via its	Channel protein	Protein motif	Main approaches <sup>a</sup>	Cell types <sup>b</sup>	References
ZO-1	PDZ-1	TRPC4	C-terminal TRL motif	cl ci	Human fetal astrocytes in culture	[15]
	PDZ-2	Cx30		cl ci at	Mouse brain and spinal cord	[98]
	PDZ-2	Cx31.9	The most C-terminal residues	cl ci	HEK 293 cells	[99]
		Cx32		cl ci	Cultured rat hepatocytes	[46]
	PDZ-1	Cx36	Four C-terminal residues (SAYV)	cl ci em at	Mouse brain, <i>HeLa</i> cells	[100]
	PDZ-1		A 14-residue C-terminal fragment	cl ci at	<i>HeLa</i> cells, $\beta$ TC-3, mouse pancreas and adrenal gland cells	[32]
				cl ci	PC12 cells	[101]
	PDZ-1			cl ci	Mouse retina, <i>HeLa</i> cells	[52]
	PDZ-1	Cx35 (orth. Cx36)	Four C-terminal residues (SAYV)	cl ci at	Goldfish brain sections, <i>HeLa</i> cells	[102]
		Cx40		cl ci	Porcine vascular endothelial cells	[103]
	PDZs	Cx43	C-terminal 5 residues	cl ci at	HEK 293 cells, rat cardiomyocytes	[24]
	PDZ-2		Extreme C-terminal	cl ci dh	COS-7, Rat-1, mink lung epithelial cells	[25]
	PDZ-2		C-terminal 5 residues	cl ci em	Rat adult ventricular myocytes	[65]
				cl ci	42GPA9 cells, rat testis lysates	[104]
	PDZ-2		C-terminal	ci at	C57B16 mouse cortical astrocytes	[71]
	PDZ-2		Last 19 C-terminal residues	nmr		[36]
	PDZ-2		CT (residues at the -3 position)	cl ci dh	MDCK cells	[35]
	PDZ-2			cl ci at	Mouse brain and spinal cord	[98]
	PDZ-2		C-terminal (amino acids 374–382)	cl at	<i>HeLa</i> cells, rat cardiomyocytes	[27]
				cl ci	Porcine vascular endothelial cells	[103]
	PDZ-2		C-terminus	cl ci	NRK and HEK 293 cells	[105]
				cl ci at	Human trophoblastic cells	[95]
				cl ci dl	<i>HeLa</i> cells	[55]
				cl ci	<i>HeLa</i> cells	[106]
				dl	Neonatal rat ventricular myocytes	[29]
				cl ci	Cultured bovine corneal endothelium cells	[91]
	PDZs	Cx43, Cx45	C-terminal 4 residues	cl ci at	ROS 17/2.8 cells	[44]
		Cx45	12 most C-terminal residues	cl ci dh	MDCK cells	[107]
	PDZ-2			cl ci at	ROS 17/2.8 cells	[108,109]
	PDZ-2	Cx46, Cx50	Most C-terminal residues	cl ci at em	<i>HeLa</i> cells	[52]
	PDZ-2		Most C-terminal residues	cl ci	Mouse lens	[110]
	PDZ-2			cl ci em at	Mouse lens	[111]
ZO-2	PDZ-2	Cx47		cl ci em at	Mouse brain, <i>HeLa</i> cells	[112]
	PDZ-1	Cx36	Four C-terminal residues (SAYV)	cl ci at	<i>HeLa</i> and $\beta$ TC-3 cells	[34]
		Cx43	C-terminal end	at	NRK cells	[113]
ZO-3	PDZ-2	Cx43	C-terminal end	cl ci dh	NRK, HEK 293T cells, heart tissues	[30]
	PDZ-1	Cx36	Four C-terminal residues (SAYV)	cl ci at	<i>HeLa</i> and $\beta$ TC-3 cells	[34]
	PDZs	Cx45	C-terminal 4 residues	cl ci dh	MDCK cells	[107]

<sup>a</sup> cl: co-localization; ci: co-immunoprecipitation; fs: co-fractionation or co-sedimentation; dh: double hybrid; dl: duolink; em: electrom microscopy immuno labeling; at: affinity techniques (pull-down, affinity binding assays, surface plasmon resonance); nmr: nuclear magnetic resonance.

<sup>b</sup> In roman characters, cells where Cx were endogenously expressed; in italics, cells where Cx were exogenously expressed, surexpressed or mutated.

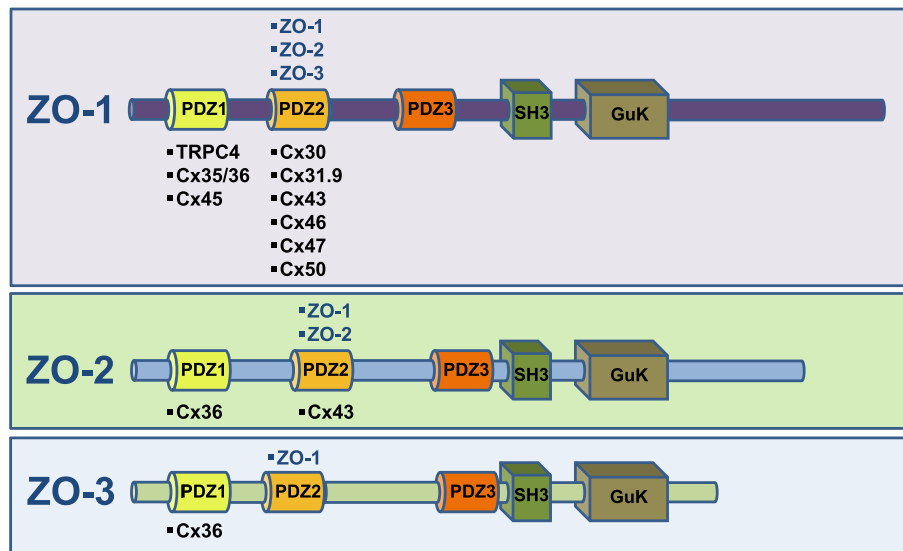
co-localization of Orai1 (the founding member of a class of “calcium release-activated” calcium channels) and ZO-1 was observed in mouse pancreatic acini [16]. The observations made in  $\text{Ca}_v1.2$ -transfected endothelial ECV cells suggested a robust interaction between ZO-1 and the voltage-gated L-type  $\text{Ca}^{2+}$  channel  $\text{Ca}_v1.2$  [17]. However, most of the reported interactions of ZO proteins with channel-forming proteins occur with connexins (Cxs), a family of structurally related transmembrane proteins that assemble to form chordate gap junctions. Each gap junction channel is made of two hemichannels, or connexons, which are themselves each constructed out of six connexin molecules. The assembly of Cxs into GJs is a multistep process. The newly synthesized Cxs are co-translationally inserted into the endoplasmic reticulum, they oligomerize to form connexons, then connexons are delivered to the cell surface either via Golgi and trans-Golgi network in small vesicles (a model largely based on the studies on Cx43 and Cx32) (see [18]) or via a Golgi-independent route for Cx26 [19] and Cx30 [20].

Hemichannels diffuse laterally within the membrane to cell-to-cell border regions, where they dock with the connexons in the contiguous cell membranes to form intercellular channels, which cluster to form a GJ plaque. Because of the relatively short half-life of Cxs (usually 1.5–5 h, see [21]), the junctional plaque is in a dynamic state, constantly remodeled through both recruitment of newly synthesized connexons to the periphery and endocytosis of older plaque components from the plaque center. Although much has been learned about these different steps, the precise spatio-temporal events and the cellular factors required for the docking of connexons, the de novo formation of a

new plaque and its life cycle remain poorly understood (see [22,23]). The activity of junctional channels is markedly influenced by intramolecular modifications (e.g. by phosphorylations of proteins) and via the formation of multiprotein complexes where pore-forming subunits bind to auxiliary channel subunits and associate with scaffolding proteins, particularly ZOs, that play essential roles in channel localization and activity. These scaffolding proteins link signaling enzymes, substrates, and potential effectors (such as channels) into multiprotein signaling complexes that may be anchored to the cytoskeleton. Protein–protein interactions play essential roles in channel localization and activity.

Cx43 was the first connexin found to interact with a ZO protein [24,25], and this interaction has been subsequently observed in a variety of cell types and with different Cx isoforms (Table 1). Cx43/ZO-1 associations do not necessarily overlap directly with whole junctional plaques, and ZO-1 has frequently been observed only at the plaque perimeter [26–28], a region termed “perinexus” [29]. The fact that ZO-2 also binds to the C-terminus of Cx43 [30] opens the possibility of competition between these ZO proteins to interact with the Cx43-PDZ-binding motif, to influence gap junctional patterning. Conversely, a competition between Cx-protein partners or even between Cxs and proteins belonging to other classes of junctional complexes (e.g. tight junctions) still widens the possibilities of adaptations of intercellular junctions to the cell needs.

As indicated in Fig. 1, connexins, except Cx36 (and its fish ortholog, teleost Cx35), which interacts with PDZ1 of ZO-1, namely Cx30, -31.9, -43, -46, -47 and -50, mainly interact with PDZ2 of ZO-1



**Fig. 1.** Domain organization of the ZO proteins. The canonical MAGUK protein binding motifs (Psd95/Discs large/Zonula, PDZ; Src homology3 domain, SH3; Guanylate kinase-like domain, GuK) are separated by “unique” regions of high sequence diversity. Some channel proteins interacting with ZO proteins (e.g. Cx32, Cx40 and Cx45) are not included in the figure since their association domain is less well described.

and, to a lesser extent, ZO-2; only Cx36 and Cx45 have been found to interact with ZO-3 (see Table 1). In Cx30-HeLa cells however, if tubulin and actin were co-precipitated with Cx30, ZO-1 protein was not [20].

In contrast with these other connexins, Cx36 contains the C-terminus amino acids Y-V, the binding motif domain present in the C-terminus of most of the claudins and reported to be responsible for their interaction with the PDZ-1 domain of ZOs [31]. A domain of 14 C-terminus amino acids (and particularly the last 4 ones) of the Cx36 sequence appeared to be required for Cx36 interaction with the PDZ-1 domain of ZO-1 since a peptide corresponding to this region showed binding capacity to the PDZ-1 domain of ZO-1 and behaved as a competitive inhibitor of Cx36/ZO-1 interaction in vitro [32]. In the retina of Cx36<sup>-/-</sup> mice, labeling of ZO-1 was only slightly reduced whereas a large reduction of ZO-2 and ZONAB (ZO-1-associated nucleic acid-binding protein) was observed [33]. These results suggest a direct interaction of Cx36 with ZO-2 and/or ZONAB rather than with ZO-1. Cultured βTC-3 and Cx36-transfected HeLa cells express ZO-2 and ZO-3 and display co-association of Cx36/ZO-2 and Cx36/ZO-3 at points of cell–cell contacts forming gap junction plaques. Molecular interaction of Cx36 with each of these ZO proteins occurs by tethering of the PDZ domain binding motif in Cx36, represented by the C-terminus SAYV amino acids in this protein, selectively to the PDZ-1 but not to the PDZ-2 or PDZ-3 domains contained in each of ZO-2 and ZO-3 [34].

Cx43 association with ZO-1 occurs via its extreme CT; Jin et al. [35] suggested that this interaction would take place through a typical Class II PDZ binding domain. Nuclear magnetic resonance (NMR) titration experiments determined that the ZO-1 PDZ-2 domain affected the last 19 amino acid residues of the CT of Cx43 [36]. Jin et al. [35] emphasized the fact that Cx31.9, Cx43, Cx46 and Cx50 exhibited similar PDZ binding motifs (DLXI) in their CT.

ZO-1 binding by Cx43 was lost when phenylalanine residues were substituted for <sup>375</sup>Pro and <sup>377</sup>Pro, showing that Cx43 localization to a gap junction implies a specific structural conformation at residues <sup>374</sup>Arg–<sup>379</sup>Asp that is also important for ZO-1 binding [37]. <sup>210</sup>Glu of ZO-1 PDZ2 was found to be a key regulatory point in Cx43 binding and phosphorylation-induced dissociation [38].

Chen et al. [39] solved the crystal structure of ZO-1 PDZ2 in complex with a 9 aa C-terminal peptide of Cx43 (NH<sub>2</sub>-RPRPDDLEI-COOH). The structure revealed that the domain swapping of ZO-1 PDZ2 generates a distinct Cx43-binding site distal to the canonical PDZ ligand-binding pocket. Importantly, the distal Cx43-binding sites in the PDZ2 dimer not only provide unprecedented interaction specificity between Cx43 and

ZO-1 PDZ2 but also function as regulatory sites for the dynamics of the Cx43/ZO-1 GJs.

Complementary biochemical studies showed that a longer peptide (12 aa, NH<sub>2</sub>-ASSRPRPDDLEI-COOH, including the completely conserved <sup>-9</sup>Ser and <sup>-10</sup>Ser) had higher binding affinity to PDZ2. Moreover, the phosphorylation of the upstream residues <sup>-9</sup>Ser or the removal of <sup>-10</sup>Ser and <sup>-9</sup>Ser from the Cx43 peptide (the nine-residue peptide) significantly reduced the amplitude of the peptide binding [39].

As <sup>-9</sup>Ser and <sup>-10</sup>Ser have been reported to be substrates of several protein kinases, including protein kinases C (PKC) and B (PKB or Akt) [40–42], Chen et al. [39] suggested that the phosphorylation of the two Ser residues (<sup>-9</sup>Ser in particular) can indeed weaken the binding between Cx43 and ZO-1 PDZ2. Considering the polyvalent properties of both Cx43 in connexons and ZO-1 PDZ2, the impact of <sup>-9</sup>Ser and/or <sup>-10</sup>Ser (corresponding to respectively <sup>372</sup>Ser and <sup>373</sup>Ser) phosphorylation of Cx43 on its ZO-1 binding (that is, decrease in the binding) is very large. Therefore, Chen et al. [39] suggested that the phosphorylation/dephosphorylation of these serine residues on Cx43 can be readily used as an on/off switch for the Cx43–ZO-1 complex formation.

Rhett et al. [43] observed an interaction of Cx43 with Nav1.5 sodium channels in the perinexus of rat cardiomyocytes; as if Cx43/ZO-1 interaction was important in this domain, Nav1.5/ZO-1 was, at best, very rare and the authors suggested that Cx43 interaction with Nav1.5 would be mutually exclusive with Cx43/ZO-1 interaction.

As ZO-1 was able to bind to a truncated Cx45 protein lacking the canonical PDZ domain-binding present at the C-tail, Laing et al. [44] suggested that Cx45 might have a large and complex binding site for ZO-1, comprising the residues between amino acid 357 and the Cx45 C-terminus, an alternative possibility being the existence of 2 distinct binding sites, one involving the C-tail and the second the amino acids proximal to amino acid 360. The authors however did not exclude either the possibility of an artifactual ZO-1/Cx45 binding or of an indirect interaction (e.g. an association of Cx45 with Cx43 bound to ZO-1). Lens Cx50 undergoes in vivo, during fiber maturation, a natural proteolytic cleavage at residue 290 [45], which would be expected to eliminate ZO-1 binding.

Cx32 was reported to interact with ZO-1 in primary rat hepatocytes [46] but Jin et al. [35] noticed that the basis for the Cx32/ZO-1 interaction differed from that of the Cx43, -40 and -45 interactions in that it did not involve a PDZ binding domain at the Cx32 C-terminus.



Structural comparison shows that the ZO-2 PDZ2 homodimers may have a similar binding pattern to that exhibited in the ZO-1 PDZ2/Cx43 complex, with however a lower affinity [47] and, indeed, ZO-2 PDZ2 associates with Cx43 (see Table 1). Interestingly, ZO-2 PDZ2 also interacts with PIP<sub>2</sub> [48].

## 5. Roles of Zonula Occludens proteins

### 5.1. Influence on the strength of cell-to-cell communication

Several studies suggest that the Cx binding to ZO proteins (particularly ZO-1) may regulate gap junction assembly in a connexin- and cell type-specific manner, at different steps.

#### 5.1.1. Hemichannel assembly

Considering the long (B140 residues) and flexible connecting sequence between the transmembrane helix and the PDZ-binding tail of Cx43, Chen et al. [39] proposed a model based on the assumption that the existence of two Cx43-binding sites in the ZO-1 PDZ2 dimer (which are  $\approx 3$  nm apart), should be able to promote and stabilize the assembly of six Cx43 molecules within one hemichannel as well as to cluster multiple hemichannels distributed on plasma membranes for the formation of functional GJs.

#### 5.1.2. Trafficking of junctional proteins

PDZ proteins, including ZOs, often exert an effect as adaptors in linking their binding partners with cellular protein trafficking machineries, thereby regulating both the biogenesis and the cellular localization of PDZ-interacting targets. Mitic et al. [49] constructed chimeric proteins containing the tail of human occludin, with or without the region that binds ZO proteins, fused to the membrane-spanning portions of Cx32. They observed that chimeras containing the ZO-binding domain of occludin targeted them to tight junction fibrils. In HeLa cells, small interfering RNAs (siRNAs) used to knock down ZO-1 levels completely inhibited normal trafficking of wild-type Cx50 and development of cell-to-cell communication [50].

#### 5.1.3. Formation and localization of gap junctional plaques

Cytoplasmic scaffold proteins appear to play key roles in the assembly of membrane specialized areas (e.g. cellular junctions, channel or receptor clusters), organizing membrane proteins into specialized membrane domains. Cytoskeletal-based perimeter fences were for example seen to selectively corral a membrane-protein sub-population of potassium channels (Kv2.1 channels) to generate stable 1–3  $\mu^2$  clusters [51]. These authors noticed that despite the stability of these microdomains, the channels retained within the cluster perimeter were surprisingly mobile, showing that the clustering did not result from a static scaffolding-based structure. ZO proteins are at the center of a network of protein interactions, linked to the actin cytoskeleton via their CT and to Cxs via their NT. In the mouse retina for example, ZO-1, interacting with Cx45 via its PDZ2 domain and with Cx36 via its PDZ-1 domain, provides for co-scaffolding of Cx45 with Cx36, forming “bihomotypic” gap junctions, with Cx45 structurally coupled to Cx45 and Cx36 coupled to Cx36 [52]. The critical role of ZO-1 in mediating this association by tethering the two Cxs within gap junctions was indicated by abolition of Cx36/Cx45 co-IP after removal of the C-terminal 4 aa residues of Cx36, required for the interaction of Cx36 with the PDZ-1 domain of ZO-1 [52]. The fact that ZO-1 and ZO-2 seem to compete throughout the cell cycle for association with Cx43 led Singh et al. [30] to suggest that the ratio of ZO-1 and ZO-2 interacting with Cx43 may influence the stability and/or stabilization of gap junctions. Connexin channels clustered in gap junctional plaques share these characteristics, where ZO-1 is preferentially localized at the periphery of the Cx43 [26–28], or Cx50 [53] plaques, suggesting that a ZO-1-actin perimeter fence could selectively corral gap junction channels (Fig. 2).

As ZO-1 can target to the periphery of Cx43 junctional plaque independently of PDZ2-mediated interactions, Hunter and Gourdie [54] put forward a targeting sequence that would initially involve ZO-1 bound to junctional complexes (possibly N-cadherin-based) adjacent to GJs, followed by a transfer of ZO-1 and its direct engagement with Cx43 at GJ edges. In Cx43-HeLa cells, actin co-immunoprecipitated with ZO-1 only in the Triton-insoluble fraction, suggesting that ZO-1, actin and Cx43 were present in the same multiprotein complex only when connexons were aggregated in detergent-resistant GJs [55]. According to Chen et al. [39], the potential multimerization of ZO-1 through its SH3 and GuK domains should further increase its Cx43 GJ formation capacity.

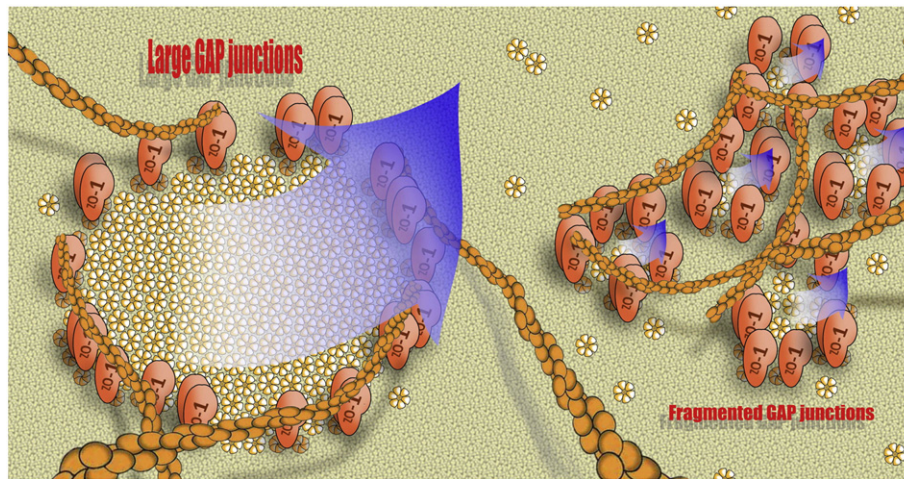
Toyofuku et al. [56] observed that functional GJs were formed even when the Cx/ZO-1 interaction was prevented. In previous studies, the expression of truncated Cx43 lacking the site of ZO-1 binding led to the formation of functional GJs [57,58]. Functional studies showed normal dye transfer between myocytes isolated from mice expressing Cx43D378stop (lacking the last five C-terminal aa residues, the binding motif for ZO-1) and unchanged electrical coupling and dye transfer in Cx43D378stop HeLa cells [59]. But haplodeficient mice expressing a CT-truncated Cx43, incompetent to interact with ZO-1, formed GJs at cardiac GJ plaques that were larger than those observed in wild-type littermates [60]. In HeLa cells, siRNAs used to knock down ZO-1 levels caused an unchecked accretion of undocked connexons at the Cx43 plaque periphery, resulting in larger GJs and fewer hemichannels [55] whereas it completely inhibited GJ formation by wild-type Cx50 [50]. In the latter study, Cx50 with PDZ-binding motif mutations neither form GJ plaques nor induce cell–cell communication, whereas the addition of a seven-amino acid PDZ-binding motif restored normal function to Cx50 lacking its entire C-terminal cytoplasmic domain.

In some cell types, gap junctions are preferentially located in specific membrane areas; in adult heart for example, Cx43 gap junctions localize to the intercalated disk (IDs) of myocytes. The link between Cx43 and  $\alpha$ -spectrin, via ZO-1, was suggested to serve to localize Cx43 at the cardiac IDs [24]. In human squamous carcinoma cells, intimacy between Cx43 and F-actin via ZO-1 seemed to be required to maintain or stabilize GJ plaques, and ZO-1 knockdown attenuated GJ formation, suggesting that direct or indirect cross-talk between Cxs and the actin cytoskeleton via ZO-1 may be essential for GJ assembly [61].

#### 5.1.4. Regulation of channel activity

Cx43-based cell-to-cell communication between Rat-1 fibroblasts was seen to be inhibited by depletion of PIP<sub>2</sub>, a compound functioning as an intermediate in the inositol 1,4,5-triphosphate and diacylglycerol (IP3/DAG) pathway, which is initiated by ligands binding to G protein-coupled receptors activating the G $\alpha_q$  subunit. PIP<sub>2</sub> is a substrate for hydrolysis by phospholipase C (PLC). Inhibiting PIP<sub>2</sub> hydrolysis kept Cx43 channels open after receptor activation [62]. As ZO-1 binds directly to the very C terminus of PLC $\beta$ 3 via its third PDZ domain, it was proposed to be a key intermediate in assembling Cx43 and PLC $\beta$ 3 into a complex, thereby facilitating regulation of Cx43 channel function via PIP<sub>2</sub> hydrolysis upon receptor activation [62]. In mouse embryonic stem cells, siRNA-caused depletion of endogenous ZO-1 reduced GJIC intensively [63]. In N/N1003A cells, Akoyev and Takemoto [64] noticed that ZO-1 was required for both PKC $\gamma$ -driven disassembly of Cx43 and effects on cell-to-cell dye transfer, that ZO-1 downregulation induced abnormal interaction of PKC $\gamma$  with Cx43 without activation by TPA (phorbol-12-myristate-13-acetate).

The F-actin cytoskeleton and actin-associated adapter proteins underlying the cell membrane appear to regulate the function of membrane complexes. In rat cardiac myocytes for example, the integrity of the actin microfilament network is essential to preserve GJ communication [28]. In such processes, the F-actin-associated adapter proteins and scaffolding proteins (particularly ZO-1) likelihood function as signal transducers.



**Fig. 2.** Cx43/ZO-1 partnership about gap junctions. Artistic view of the perimeter fence attached to the cortical cytoskeleton that ZO-1 seems to form around the clustered intercellular channels. The fragmentation of large gap junction plaques into small elements increases the Cx43/ZO-1 interaction.

#### 5.1.5. Degradation of channel proteins

Earliest electron microscopy observations revealed that gap junction fragments were degraded by internalization of a double-membrane structure termed “annular junction” into one of the two cells, where subsequent lysosomal or proteasomal degradation occurred, except in some cases where the connexons were recycled. ZO-1 was suggested to confer specificity to actin-based contractile processes via its ability to simultaneously interact with Cx43 and cytoskeleton [65].

In 42GPA9 Sertoli cells, lindane ( $\gamma$ -hexachlorocyclohexane, a lipid-soluble pesticide) enhanced the endocytic process, causing an intracytoplasmic delocalization of the membranous signal for both Cx43 and ZO-1, while localization of other tight junction proteins (e.g. occludin or claudin) was not affected [66]. In the same cell line, Gilleron et al. [67] noticed that if ZO-1 was usually localized on both sides of the gap junction plaque, its presence became restricted to one side of the junctional plaque during the endocytic GJ internalization and the formation of annular junctions, suggesting that the dissociation of ZO-1 from Cx43 – preferentially on one side of the junctional plaque – had a prominent role in GJ endocytosis. In HeLa cells stably expressing tagged Cx43 (Cx43-YFP, Cx43 tagging being known to prevent ZO-1 binding), GJ internalization did not occur in response to inflammatory mediators, showing that ZO-1/Cx43 interaction is required for GJ internalization [68].

42GPA9 Sertoli cells endogenously express Cx43 but transfection of tagged Cx33 affected gap junctional communication by intracellular association with Cx43 and sequestration of the complex Cx43/Cx33 in the early endosomes [69]. In wild-type Sertoli cells transfected with Cx33-DsRed2 and Cx43-green fluorescent protein vectors, heteromeric Cx33/Cx43 hemi-channels were formed and incorporated into gap junction plaques, but such plaques appeared unstable at this level and were rapidly internalized [70]. The authors suggested that the heteromeric Cx33/Cx43 complex may interact less than the homomeric Cx43 complex with ZO-1 because of the lack of binding site for ZO-1 on Cx33. As among its described functions, ZO-1 is known to secure Cxs in gap junction plaques at the cell–cell boundary, the reduced association of ZO-1 with the Cx33/Cx43 complex could then explain the Cx33/Cx43 gap junction plaque instability.

c-Src appears to influence the stability of GJ plaques via modifications in the Cx43/c-Src/ZO-1 complex, facilitating the GJ internalization [67,71,72] and subsequent degradation of annular junctions [73]. According to Akoyev and Takemoto [64], the close proximity of the <sup>368</sup>Ser/PKC $\gamma$  phosphorylation site to the ZO-1 PDZ-binding domain on the Cx43-CT suggests that ZO-1/Cx43 effects and PKC $\gamma$  effects may be connected, ZO1 being able to modulate the interaction of PKC $\gamma$  with Cx43 and, thus, the PKC $\gamma$ -driven gap junction disassembly

of Cx43. Consequences of the binding of the c-Src SH3 domain to Cx43 and to Cx40 differ on the interactions of these Cxs with ZO-1. It can indeed disrupt the Cx43/ZO-1 interaction, leading to down-regulation of GJIC [36,56,71] whereas it does not affect the Cx40-CT/ZO-1 PDZ-2 domain complex [74].

#### 5.2. Effects on other biological processes

Besides the transmembrane diffusion of small molecules and metabolites that support tissue homeostasis and electrical and chemical signaling, gap junction proteins are, via their selective protein–protein interactions, involved in the control of cell growth, proliferation, apoptosis, and would serve as tumor suppressors. In the model proposed by Solan and Lampe [75] for example in normal rat kidney cells during their progression from G0/G1 through S phase and into mitosis, Cx43 is at first found predominantly at the plasma membrane, assembled into “typical” gap junction plaques, associated with both ZO-1 and ZO-2, at the plasma membrane. Then the assembly of Cx43 into plaques becomes less efficient, accompanied by a shift in subcellular Cx43 localization and a decreased co-localization with ZO-1 while the ZO-2 interaction is maintained. When cells enter mitosis, GJIC is interrupted and Cx43 is predominantly intra-cytoplasmic [75].

ZO proteins are known to be engaged in the transmission of signals from the plasma membrane to the nucleus to regulate gene expression. ZO-1 is associated with the transcription factor ZONAB [76], while ZO-2 is with the transcription factors Jun, Fos and C/EBP [77]. The association of ZOs with GJ proteins results in the membrane sequestration of transcription factors and hence the inhibition of their transcriptional activity.

### 6. Physiological consequences

#### 6.1. Physiological modulation of the ZO-1–connexin partnership

In the mammalian retina, Puller et al. [53] noticed that ZO-1 formed margins around GJ and was closely associated with glutamate receptors, and suggested that that glutamate released from the photoreceptors might play a role in modulating the conductance of electrical synapses in the outer plexiform layer of the retina.

In rat cardiac myocytes, the RhoA signaling pathway dynamically modulates the permeability of Cx43-made channels: RhoA activation was seen to rapidly enhance the strength of cell-to-cell communication but reduce Cx43/ZO-1 co-localization whereas RhoA inhibition had opposite effects, GJIC down-regulation and enhancement of Cx43/ZO-1 co-localization [28]. In mouse primary cultures of astrocytes, a

population of Cx43 (likely junctional Cx43) was found associated to ZO-1/ZO-2/occludin multiprotein complexes; acute treatments of cells with endothelin-1 or sphingosine-1-phosphate induced both an increase in the amount of Cx43 linked to these complexes and a drastic GJIC inhibition [78]. In mouse embryonic stem cells, siRNA-caused depletion of endogenous ZO-1 reduced GJIC intensively [63].

## 6.2. ZO-1–connexin interactions in pathological conditions

As GJ channels are conduits for propagation of electric activity, the cardiac specific pattern (GJs preferentially localized to the long ends of the cells, in IDs) led to hypothesize that this localization was important for the physiological anisotropic conduction of the heart. In acquired adult heart disease (as ischemic heart disease or heart failure), a gap junction remodeling is observed, with alterations in the distribution of gap junctions and the amount and type of expressed Cxs, a “lateralization” of GJs, suspected to be involved in alteration of conduction (for review see for example [79]).

This remodeling has been suggested to result from alterations in Cx43/ZO-1 interaction [80,81]. ZO-1 forms with N-cadherin a multiprotein complex which, according to Palatinus et al. [82], would be a key determinant of stable localization of both adherens junctions and gap junctions at the ID. This is consistent with previous observations that cardiac-specific deletion of N-cadherin led to alteration in Cx40 and Cx43, disassembly of the ID structure and conduction slowing and arrhythmogenesis in adult mice [83,84]. Hesketh et al. [85] observed that GJs formed between the lateral membranes of cardiomyocytes with increased frequency in a canine model of failing heart did not co-localize with ZO-1 and cadherin. In transverse sections of normal dog epicardium, ZO-1 extensively covered the ID region and Cx43 was found primarily around the ends of the myocytes but after coronary occlusion, Cx43 localized to lateral membranes, causing decreased conduction velocity and formation of an arrhythmogenic substrate, whereas disk regions remained comprised of ZO-1 alone [81].

A mimetic peptide corresponding to the last 9 aa of the Cx43 tail caused an increase in GJ plaque size mainly ascribed to unregulated accretion of connexons from nonjunctional pools, with a possible contribution from increased plaque fusions [27]. In a model of cryoinjury of mouse left ventricle, treatment with a peptide incorporating the last 9 aa of the Cx43-CT decreased stimulated arrhythmias such as ventricular tachycardia or fibrillation. Together with these effects on cardiac electrical function, the peptide reduced ZO-1/Cx43 co-localization and GJ lateralization in arrhythmia-prone tissues of the injury border zone [86]. However, the peptide effects on GJ remodeling may not have only resulted from effects on ZO-1/Cx43 interaction, the authors identified a novel peptide target, PKC- $\epsilon$ . The peptide increased PKC- $\epsilon$  phosphorylation of a consensus target on the Cx43-CT, the serine residue <sup>368</sup>Ser. Consistent with the *in vitro* results, levels of phosphorylated <sup>368</sup>Ser in the injury border zone were elevated significantly above controls within hours of peptide exposure, suggesting a potential regulatory interplay between PKC- $\epsilon$  and ZO-1 at the Cx43-CT [86]. In a rat model of pressure overload hypertrophy, late stages were found to be associated with a marked dephosphorylation of Cx43 accompanying loss of association with ZO-1 and pronounced conduction delays [87].

Matrix metalloproteinases (MMPs) contribute to left ventricular remodeling after myocardial infarction; MMP-7, abundant in cardiomyocytes and macrophages, was found capable of mediating Cx43 cleavage, releasing small free peptides derived from the extreme C-terminus of Cx43. This truncation did not impede connexon formation but such connexons were not able to interact with ZO-1 [88].

Cancer was the first pathology proposed to be associated with malfunction of direct cell-to-cell communication. Many tumors are indeed deficient in gap junction function for abnormal connexins (induced e.g. by Cx mutation, mRNA decrease or aberrant of Cxs), the abnormal distribution of Cxs (in the cytoplasm rather than into membrane) and the abnormal regulation by other proteins (for review, see [89]). In the

SerW3 Sertoli cell line, DDT (1,1,1-trichloro-2,2-bis(p-chlorophenyl) ethane), a non-genomic carcinogen, was seen to induce abnormal disintegration of the GJ plaque and internalization of both Cx43 and its partners (ZO-1 and N-cadherin), through the formation of clathrin-dependent endocytic vacuoles [90]. Exposure of 42GPA9 Sertoli cells to a non-genomic carcinogen (lindane) induced a GJ plaque internalization that was associated with an increased interaction of c-Src with Cx43 and a concomitant decreased Cx43/ZO-1 association, with an efficient inhibition of intercellular coupling [67]. In contrast, cultured bovine corneal endothelial cells exposed to DNA-damaging agents (e.g. mitomycin C), exhibited Cx43 accumulation in large GJ plaques, with reduction of ZO-1 binding, and displayed increased plaque stability [91].

Applied to acute skin lesions, the mimetic peptide corresponding to the last 9 amino acids of the Cx43 tail was reported [92] to accelerate wound healing while reducing scar formation in a mouse model of cutaneous injury, but the peptide effects on wound healing were in some respects not similar to those reported for Cx43 antisense treatment [93], making unclear whether the Cx43 antisense or the peptide designed to target ZO-1 acted via related mechanisms.

Xu et al. [94] rescued the embryonic lethality of ZO-2 knockout mice by injecting ZO-2(–/–) embryonic stem cells into wild-type blastocysts to generate viable ZO-2 chimeras. Adult chimeras presented a set of phenotypes in different organs; in particular, male ZO-2 chimeras show reduced fertility and pathological changes in the testis. The pre-dominant localization of Cx43 to the region of the blood–testis barrier for example was perturbed, and the protein was readily detected in the adluminal domain. Because Cx43 is essential for the initiation and maintenance of spermatogenesis, Xu et al. [94] suggested that its perturbed localization in ZO-2 chimera tubules may contribute to the reduced fertility of ZO-2 chimera males.

In human trophoblastic primary culture, gene inactivation for ZO-1 using siRNA caused a significant decrease in Cx43, suggesting that ZO-1 expression may be a prerequisite for Cx43 expression. And since Cx43 expression is associated with trophoblastic cell differentiation, ZO-1 siRNA treatment resulted in a large number of non-aggregated mononuclear cells [95].

## 7. Conclusions and perspectives

The cytoplasmic adapter proteins ZOs are capable of assembling a variety of membrane-associated proteins and signaling molecules in short-lived functional units. Their requirement (particularly of ZO-1) for the formation of GJ plaques and the establishment of intercellular coupling varies between Cxs. For Cx50-mediated GJIC, an intact PDZ-binding motif and ZO-1 are indispensable but has much less importance in cell-to-cell communication via Cx46 channels [50], and large GJ plaques are formed even when the Cx43/ZO-1 interaction is prevented [55–60]. In this case, the area surrounding gap junctional plaques seems populated by unpaired connexons and ZO-1 appears to regulate the size of gap junction plaques by limiting the access of ZO-1-associated Cx43 into pre-existing gap junctions. Moreover, at least for Cx43, the functional interaction of the C-terminus of the connexin with ZO-1 PDZ2 is essential for the GJ dynamic regulation.

The PDZ domains of ZO proteins play key roles in the assembly and regulation of cellular signaling pathways. Moreover, their function can be tuned by various means such as allosteric effects, disulfide bond formation, auto-inhibition, competitive binding and phosphorylation of PDZ domains and/or ligands, which pose PDZ domains as dynamic regulators of cell signaling.

It is noteworthy that among gap junction proteins, only some Cxs have up to now been reported to interact with ZO proteins. In the animal kingdom indeed, three families of GJ proteins are known, the well characterized Cxs in deuterostomes (Echinodermata, Urochordata, Cephalochordata and Vertebrata), the innexins (“invertebrate connexins”, Inxs) in protostomes (Nematoda, Mollusca,



Platyhelminthes, Arthropoda, Annelida) at first, then search of the human genome revealed three *Inx*-related genes, and three corresponding proteins, termed pannexins (Panxs), were identified (see [96]). Neither *Inxs* nor Panxs were until now seen to interact with ZO proteins, but pannexin 1 directly interacts with actin (preferentially F-actin; [97]).

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